Development of a tonic form of synaptic inhibition in rat cerebellar granule cells resulting from persistent activation of GABA$_A$ receptors

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1. To investigate the origin and functional significance of a recently described tonic GABA$_A$ receptor-mediated conductance in cerebellar granule cells we have made recordings from cells in cerebellar slices from rats of different ages (postnatal days P4 to P28).

2. During development there was a dramatic change in the properties of GABA-mediated synaptic transmission. The contribution to GABA$_A$ receptor-mediated charge transfer from the tonic conductance (G_{GABA}), relative to that resulting from discrete spontaneous postsynaptic currents (sPSCs), was increased from 5% at P7 to 99% at P21. G_{GABA} was reduced by bicuculline, tetrodotoxin and by lowering extracellular Ca^{2+}, and was initially present only in those cells which exhibited sPSCs.

3. At P7 sPSCs were depolarizing, occasionally triggering a single action potential. By P18 the GABA reversal potential was shifted close to the resting potential and G_{GABA} produced a shunting inhibition. Removal of G_{GABA} by bicuculline increased granule cell excitability in response to current injection.

4. This novel tonic inhibition is present despite the low number of Golgi cell synapses on individual granule cells and appears to result from 'overspill' of synaptically released GABA leading to activation of synaptic and extrasynaptic GABA$_A$ receptors.

Sensory information is transmitted to the cerebellum by mossy fibres and relayed via granule cells to Purkinje cells, which generate the final output of the cerebellar cortex. Granule cells contribute to a massive divergence of this sensory input, yet a key feature of theories regarding cerebellar function is that only a small fraction of available inputs should be effective at any one time (Marr, 1969; Gabbiani, Midtgaard & Knöpfel, 1994). The necessary filtering of mossy fibre input is thought to result from the GABA-mediated inhibition of granule cells provided by Golgi cell innervation (Gabbiani et al. 1994). At most GABAergic synapses the release of transmitter usually results in the synchronous activation of ligand-gated ion channels, giving rise to fast events which operate in the millisecond time range. While granule cells exhibit such GABA$_A$ receptor-mediated postsynaptic currents (PSCs), recent experiments have identified an additional tonic conductance that appears to arise from persistent activation of these receptors (Kaneda, Farrant & Cull-Candy, 1995; Tia, Wang, Kotchabhakdi & Vicini, 1996; Wall & Usowicz, 1996). The functional significance of this tonic conductance is unknown. In this study we have investigated its development, origin

and effect on granule cell excitability. Our results suggest that this conductance arises following the formation of synaptic contacts and that it results from 'overspill' of synaptically released GABA leading to the continuous activation of GABA$_A$ receptors, which may be both synaptic and extrasynaptic (Nusser, Roberts, Baude, Richards & Somogyi, 1995). Although relatively few channels are open at any one time, their activation is sufficient to produce a novel form of shunting inhibition capable of dynamically regulating granule cell excitability.

METHODS

Whole-cell recording

Cells were viewed with Nomarski differential contrast optics (×1000; Axioscope FS, Zeiss). Patch-pipettes were pulled from thick-walled glass (GC150F-7·5, Clark Electromedical), coated with Sylgard (no. 184, Dow Corning) and had a resistance of 5–10 MΩ. The pipette solution contained (mM): CsCl, 140; NaCl, 4; CaCl$_2$, 0·5; Hepes, 10; EGTA, 5; Mg-ATP, 2 (adjusted to pH 7·3 with CsOH). The bathing solution contained (mM): NaCl, 125; KCl,

2·5; $CaCl_2$, 2; $MgCl_2$, 1; $NaHCO_3$, 26; NaH_2PO_4, 1·25; glucose, 25 (pH 7·4 when bubbled with 95% O_2 and 5% CO_2). No correction was made for the estimated liquid junction potential of 3·9 mV (calculated from the Generalised Henderson equation using Axoscope 1.1; Axon Instruments). Current recordings were obtained at 22–25 °C (Axopatch 1D or Axopatch 200A; Axon Instruments). Input resistance, series resistance and cell capacitance were determined from the current response to a 10 mV hyperpolarizing voltage step; currents were filtered at 50 kHz (−3 dB, 4-pole Bessel filter) and digitized at 200 kHz. Series resistance (25–40 MΩ) and capacitance (2·6–3·7pF) were similar to values described previously (Kaneda *et al.* 1995) and were similar at all ages. Input resistance decreased significantly with age, from 11·2 ± 1·9 GΩ at P7 ($n = 28$) to 4·6 ± 1·4 GΩ at P21 ($n = 10$). All other data were stored on digital audiotape (DC to 20 kHz). For analysis, currents were filtered at 2 kHz (−3 dB, 8-pole Bessel filter) and digitized at 10 kHz. Synaptic currents were identified visually from the digitized record and analysed using software written by Stephen Traynelis (Emory University, Atlanta, GA, USA). The holding current and current variance for each record was calculated from a 200 ms epoch containing no synaptic events. Measurements of GABA-mediated sPSC kinetics and background conductance, as shown in Fig. 1, were made in the presence of 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX; 5 μM) and D-2-amino-5-phosphono-pentanoic acid (AP5; 10 μM) to block non-*N*-methyl-D-aspartate (non-NMDA)- and NMDA-type glutamate receptors. Strychnine (300 nM) was also included to block glycine receptors (Kaneda *et al.* 1995). GABA-mediated sPSCs reversed at +8 mV ($n = 10$), close to the estimated Cl⁻ reversal potential of +2 mV (given the predicted junction potential), and this value was used in the calculation of all conductances. Charge transfer resulting from tonic $GABA_A$ receptor activation was calculated from the measured conductance in the absence and presence of bicuculline. Charge transfer via PSCs was calculated from the integral of their averaged waveforms and their mean frequency.

Perforated-patch recording with gramicidin

To avoid altering the normal intracellular Cl⁻ concentration during current-clamp recordings these were made using the gramicidin-perforated-patch technique, as described by Kyrozis & Reichling (1995). The pipette solution contained (mM): KCl, 145; Hepes, 10; EGTA, 5; $CaCl_2$, 0·1 (pH 7·3 with KOH). Pipettes were tip-filled with this solution then back-filled with one containing 6–9 μg ml⁻¹ gramicidin. Series resistance was continuously monitored in voltage-clamp mode and the switch to current clamp (I_{fast} setting, Axopatch 200A) was made once this was < 100 MΩ (20–60 min after forming seal). No correction was made for the estimated liquid junction potential of 3·3 mV. Except where stated, all current-clamp recordings were obtained in the absence of glutamate and glycine receptor antagonists. All values are expressed as the mean ± S.E.M. Statistical comparisons were made using Student's *t* test and non-parametric randomization or sign tests; differences were considered significant at $P < 0.05$.

RESULTS

GABA-mediated sPSCs were first observed at P4 and were present in most granule cells by P7 (88 of 110 cells). sPSCs persisted in the presence of glutamate receptor antagonists, but were abolished by the $GABA_A$ receptor antagonists bicuculline or SR-95531 at all ages (10 μM; $n = 29$). In those cells with sPSCs the frequency of events was not significantly

different between ages: 0·8 ± 0·1 Hz at P7 ($n = 32$) and 1·3 ± 0·8 Hz at P21 ($n = 6$). In the presence of TTX (300 nM), miniature PSCs were easily identified but were extremely infrequent (frequency reduced from 0·91 ± 0·34 to 0·07 ± 0·03 Hz, $n = 5$, at P14). Thus, the majority of $GABA_A$ receptor-mediated sPSCs occur as a result of action potentials in presynaptic Golgi cells.

During development sPSCs became progressively smaller and briefer (Fig. 1*A* and *B*). Peak conductances were 1·1 ± 0·1 nS at P7 ($n = 16$), 0·9 ± 0·1 nS at P14 ($n = 18$), and 0·6 ± 0·1 nS at P21 ($n = 4$). At all ages the rise times of averaged currents were < 0·5 ms. PSC decays were best described by three exponentials at P7 and by two or three exponentials at P14 and P21 (not shown). To simplify comparison across ages we measured the 62% decay times; these were 31·2 ± 2·2 ms at P7, 15·9 ± 1·9 ms at P14 and 8·0 ± 0·5 ms at P21. Overall, there was an 8-fold reduction in charge transfer by discrete events during the first 3 weeks of development (Fig. 1*D*). A similar speeding of sPSC decay was recently reported by Tia *et al.* (1996) and was attributed, in part, to a change in the subunit composition of synaptic $GABA_A$ receptors. As is apparent from Fig. 1*A*, the developmental change in kinetics and amplitude of sPSCs was accompanied by a dramatic increase in current noise and 'background' conductance (G_{GABA}) which was blocked by bicuculline (Fig. 1*C* and *D*). The net effect of these changes was that the total charge transfer via sPSCs, relative to that resulting from G_{GABA}, was reduced from 95% at P7 to only 1% at P21.

It seemed possible that G_{GABA} could reflect leakage of GABA from damaged cells, and that this may be more prevalent in slices from older animals. However, several pieces of evidence suggest that this is not the case. Firstly, G_{GABA} was dependent on the presence of functional GABAergic synapses. At P14, elevated background noise was observed only in those cells that exhibited sPSCs (G_{GABA} 100 ± 16 pS, $n = 22$). Cells lacking sPSCs, whose dendrites may not have formed synaptic contacts, showed very little background noise (G_{GABA} 3 ± 4 pS, $n = 11$) but responded to bath-applied GABA (peak conductance 3·6 ± 0·5 nS, $n = 3$, 100 μM). It therefore seems unlikely that G_{GABA} arose from a general increase in the concentration of extracellular GABA (although, at present, we cannot exclude the possibility that in those cells exhibiting sPSCs the EC_{50} for GABA was decreased). Secondly, at this age the magnitude of the background noise was clearly dependent on the level of transmitter release. Thus, reducing the sPSC frequency by the addition of TTX, or by lowering external Ca^{2+}, reduced G_{GABA} by ~85% (5 and 4 cells, respectively; Fig. 1*C*; see also Kaneda *et al.* 1995). Conversely, the non-NMDA glutamate receptor antagonist CNQX, which has been reported to increase the firing of GABAergic interneurones (McBain, Eaton, Brown & Dingledine, 1992), significantly increased both sPSC frequency and G_{GABA} (frequency, 0·6 ± 0·2 to 2·5 ± 0·4 Hz; G_{GABA}, 176 ± 96 to 347 ± 119 pS; P14,

$n = 14$), without affecting the time course or amplitude of sPSCs ($n = 4$). Together these data suggest that G_{GABA} results from the action of synaptically released GABA.

To determine the functional effect of the different patterns of GABA$_A$ receptor activation seen during cerebellar development we made voltage recordings at different ages using the gramicidin-perforated-patch technique, which avoids disruption of the internal Cl$^-$ concentration (Kyrozis & Reichling, 1995). In young cells (P7), discrete spontaneous synaptic potentials were present, which were depolarizing ($\sim$15 mV) at the resting membrane potential ($-58\cdot7 \pm 6\cdot0$ mV, $n = 5$) and were blocked by bicuculline (Fig. 2A). Depolarizing GABA responses have been reported in several other neuronal types at embryonic and early postnatal stages and are thought to reflect an immature state of Cl$^-$ distribution (see Cherubini, Gaiarsa & Ben-Ari, 1991, for review). In granule cells the depolarizing GABA-mediated events were occasionally sufficient to initiate firing (Fig. 2B)

but the majority of cells at this age were incapable of supporting action potentials (Fig. 2C) and those that did fire gave only a single spike even with large depolarizing current injections. This is consistent with the small amplitude of Na$^+$ currents at this age (data not shown). Together with the immature state of mossy and parallel fibre connections at this age, these data suggest that early GABA-mediated excitatory synaptic events do not contribute to circuit behaviour but may be involved in other aspects of granule cell maturation. In older animals (P18–21) discrete spontaneous synaptic potentials were not apparent at the resting potential ($-71\cdot2 \pm 4\cdot5$ mV; $n = 6$). The absence of synaptic events in these recordings was due to a developmental shift in the GABA reversal potential (E_{GABA}) close to the resting potential. At P7, E_{GABA}, as determined by application of GABA itself or the selective GABA$_A$ agonist muscimol, was $-24\cdot9 \pm 2\cdot9$ mV ($n = 5$) but in older animals (P18–21) E_{GABA} was $-62\cdot9 \pm 4\cdot8$ mV ($n = 6$) (Fig. 2B). While GABA-
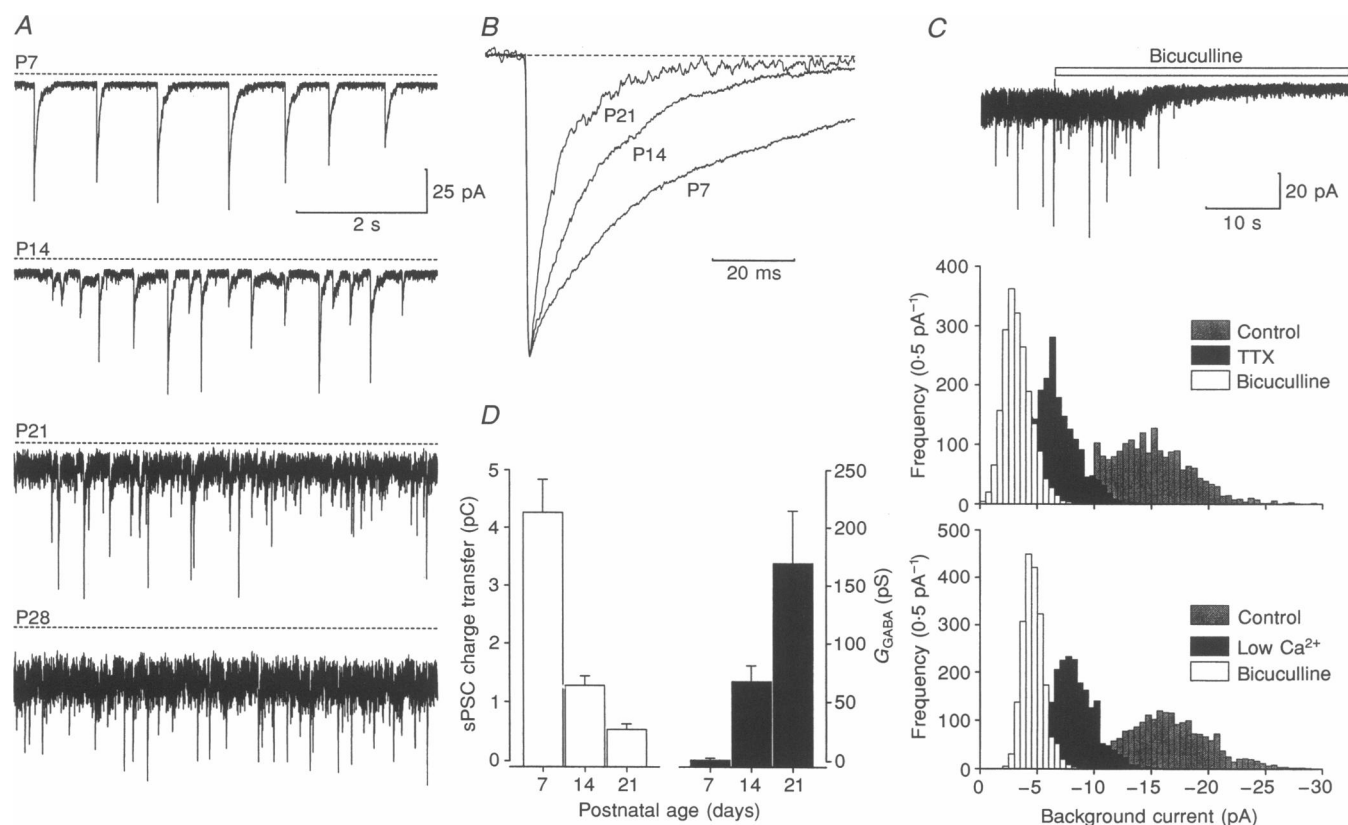

Figure 1. Properties of GABA-mediated transmission in granule cells of different ages

A, spontaneous PSCs at P7, 14, 21 and 28 (pipette potential, -70 mV) recorded in the presence of CNQX, AP5 and strychnine. The dashed lines indicate the zero current level. B, superimposed averaged PSCs at P7, 14 and 21 (50, 31 and 100 events). Traces are normalized to the peak and show the first 80 ms of the decay. The dashed line indicates the pre-event baseline. C, upper trace shows the effect of bicuculline on sPSCs and noise (10 μM; P14, -70 mV). The all-point amplitude histograms illustrate the effect of TTX (300 nM), low Ca^{2+} (0·5 mM Ca^{2+}, 5 mM Mg^{2+}) and bicuculline (10 μM; two different cells, P14, -70 mV). D, histograms showing the developmental decrease in charge transfer ($\square$) by discrete sPSCs and the increase in the bicuculline-sensitive background conductance ($\blacksquare$, G_{GABA}). Data are from 7–33 cells. At each age, G_{GABA} was calculated from all cells recorded, irrespective of the presence or absence of sPSCs. Error bars indicate S.E.M.

mediated sPSCs in young animals are excitatory, the later shift in E_{GABA} close to the resting potential suggested that $GABA_A$ receptor activation in older animals may lead primarily to a shunting inhibition.

The persistent activation of $GABA_A$ receptors in older granule cells may be sufficient to produce a tonic effect on cell excitability. We tested this idea directly with gramicidin-perforated-patch recording. As shown in Fig. 2C, depolarization of P21 cells by current injection invariably triggered overshooting action potentials, with properties similar to those previously observed with conventional whole-cell recording (D'Angelo, De Filippi, Rossi & Taglietti, 1995). To determine the effect of tonic $GABA_A$ receptor activation on cell excitability, current injection was repeated in the presence of bicuculline (Fig. 3A). In control conditions at P18, action potentials were first elicited by current injection of 6–12 pA (mean 8.5 ± 1.5 pA, $n = 4$). Following the application of bicuculline (10 μM), consistently less current was required (range 2–6 pA; mean 3.5 ± 0.9 pA, $n = 4$) and the input–output relationship exhibited a clear shift to

the left (Fig. 3B and C). Due to the relatively infrequent occurrence and brevity of sPSCs, less than 10 % of the total GABA-mediated charge transfer occurs through discrete synaptic events at this age. Thus, the increased excitability in bicuculline primarily reflects the removal of the background $GABA_A$ receptor-mediated conductance, G_{GABA}, rather than the block of sPSCs in the recorded cell.

DISCUSSION

Our data provide evidence for an unusual form of tonic inhibition in cerebellar granule cells which arises from the persistent activation of $GABA_A$ receptors. Although the resulting background conductance is ultimately dependent on impulse-linked transmitter release, it involves the steady activation of receptors and the opening of relatively few channels. For example, at P14 G_{GABA} corresponded, on average, to the superimposed opening of only three to six channels (based on single-channel conductances of 16 and 28 pS; Kaneda *et al.* 1995). In contrast, miniature PSCs (in
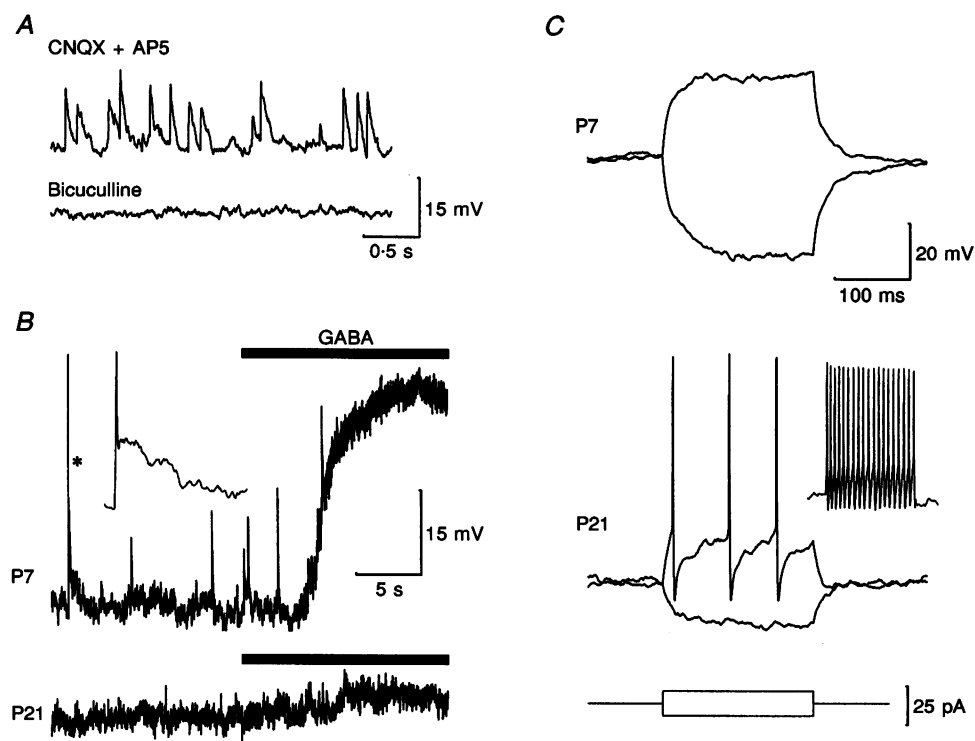

Figure 2. Voltage recordings with intact intracellular Cl⁻

A, current-clamp records at P7 (resting potential −55 mV) showing spontaneous depolarizing potentials which persist in CNQX (5 μM) and AP5 (10 μM; upper trace) but which are abolished by bicuculline (10 μM; lower trace). *B*, voltage recording from a P7 cell (upper trace); the resting potential was −74 mV and application of GABA (10 μM; bar) caused a large depolarization (56 mV). Inset shows an enlargement of a spontaneous depolarizing potential (*) which gave rise to an action potential. The lower trace is from a P21 cell; the resting potential was −85 mV and GABA caused a 3 mV depolarization. *C*, the upper panel shows a representative response at P7 to depolarizing and hyperpolarizing current injections of 8 pA. The lower panel shows a response at P21 to the same current injections. The inset shows the response in the same cell to injection of 20 pA which caused rapid, non-accommodating, firing of action potentials (mean frequency 95 Hz).

the presence of TTX) had a peak conductance of 500 ± 40 pS ($n = 4$), which represents the opening of eighteen to thirty-two channels (Kaneda *et al.* 1995). Moreover, variance analysis of the background noise yielded a conductance estimate which corresponded to that of a single channel, rather than a single quantum (see Kaneda *et al.*1995). Thus, unlike the situation in hippocampal (Otis, Staley & Mody, 1991) and cortical (Salin & Prince, 1996) neurones, where a bicuculline-sensitive background conductance can be ascribed to the summation of sPSCs occurring at high frequency, in granule cells G_{GABA} does not result from overlapping quantal events. Rather, G_{GABA} in these cells appears to reflect the activation of GABA$_A$ receptors in the continuous presence of a relatively low concentration of transmitter.

The persistent activation of GABA$_A$ receptors may reflect an extreme form of intersynaptic diffusion of transmitter, or 'overspill', between neighbouring synapses (see Barbour, Pouzat & Häusser, 1996, for review). This could arise because of the unusual arrangement of synaptic inputs on to the granule cell, which are restricted to the terminal portions of the dendrites within specialized glomerular structures (Hámori & Somogyi, 1983; Jakab & Hámori, 1988). In the adult, individual granule cell dendrites receive only two to three contacts from Golgi cell axonal varicosities, but the glomerulus contains up to 150 such synapses on dendrites from fifty different granule cells (Jakab & Hámori, 1988). As the glomerulus is enclosed within a glial sheath (Hámori & Somogyi, 1983), synaptically
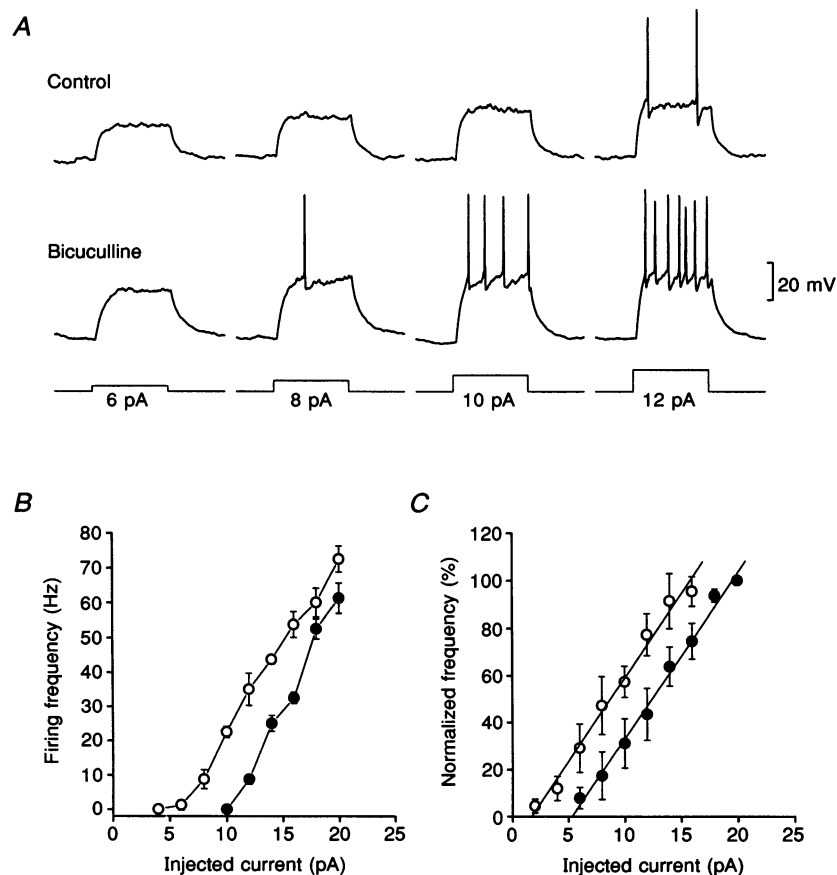

Figure 3. Voltage records showing the effect of bicuculline on granule cell excitability (P18)

A, in control conditions (upper traces) spiking was initiated by a 12 pA depolarizing current pulse (step; 200 ms). In bicuculline (10 μM; lower traces) spiking first occurred with 8 pA injection. In this cell, input resistance (determined from the slope of the current–voltage relationship during hyperpolarizing current injections) increased from 2·1 to 2·7 GΩ in the presence of bicuculline. On average, in 4 cells, there was a 22% increase in input resistance in bicuculline. *B*, mean spike frequency *versus* injected current for a single cell (same cell as in *A*). For panels *B* and *C*: ●, control; ○, bicuculline. Data are the mean of 4 trials. In this case the threshold was shifted from 12 to 6 pA with little change in the 'slope' of the relationship. Linear regression analysis of data from 4 cells indicated no significant change in this slope (5·45 ± 0·99 to 5·42 ± 0·71 Hz pA^{-1}) but a significant difference in the response to current injection ($P = 0.0019$, two-tailed sign test). *C*, normalized input–output relationships from 4 cells. Continuous lines are linear regressions with slopes of 7·01 and 7·08 (% maximal frequency pA^{-1}) and intercepts of 5·3 and 1·7 pA in control and bicuculline, respectively. The maximal spike frequency (70 ± 12 Hz) was taken as that seen under control conditions in response to 20 pA injection. In *B* and *C* error bars indicate S.E.M.

released GABA may persist and activate synaptic as well as extrasynaptic GABA$_A$ receptors (Kaneda *et al.* 1995), both of which are known to be present on the intraglomerular dendritic membrane (Nusser *et al.* 1995). Overall, our data are consistent with this model and suggest that GABA release from Golgi cells may modulate granule cell excitability both phasically (via discrete PSCs) and tonically via the generation of a persistent background conductance, the relative contributions of these two processes changing during development. The progressive development of background noise after P7, despite the expression of GABA transporters by Golgi cells and glia surrounding the glomerulus (Itouji, Saki, Tanaka & Saito, 1996), is likely to reflect the increasing number of GABA-releasing terminals within the glomerulus (Hámori & Somogyi, 1983). Diffusion of GABA may also become progressively restricted during glomerular development such that GABA remains in the cleft for a longer period. Increased GABA$_A$ receptor affinity, resulting from predicted changes in receptor subunit composition (Laurie, Seeburg & Wisden, 1992; Ducic, Caruncho, Zhu, Vicini & Costa, 1995; Saxena & Macdonald, 1996), and increased GABA$_A$ receptor number (Kaneda *et al.* 1995) may also contribute. Although uptake can regulate transmitter overspill in other brain areas (see Barbour *et al.* 1996, for references), recent experiments suggest that inhibition of GABA uptake is without effect on G_{GABA} in granule cells from adult rats (Wall & Usowicz, 1996).

In the mature cerebellum it appears that tonic GABA$_A$ receptor activation provides a shunting inhibition which may be capable of effectively filtering mossy fibre input before this sensory information is relayed to Purkinje cells (Marr, 1969; Gabbiani *et al.* 1994). Within the cerebellum, Purkinje cells receive more than 100 000 parallel fibre contacts, yet, in young animals at least, activation of only fifty granule cells is sufficient to cause Purkinje cell firing (see Barbour, 1993). Compartmental modeling of granule cells (Gabbiani *et al.* 1994) suggests that even a low value of G_{GABA} (100 pS), as seen here, would be effective in modifying the way in which the cells respond to excitatory inputs, increasing the likelihood that they fire only in response to closely timed mossy fibre signals. Such tonic inhibition, being subject to modulation by changes in mossy fibre activity, would dynamically influence granule cell excitability to maintain this reliance on synchronized input from independent mossy fibres, even at high input frequencies (Gabbiani *et al.* 1994; D'Angelo *et al.* 1995). *In vivo*, Golgi cells fire at average frequencies of ~10 Hz and exhibit brief periods of higher activity during certain motor actions (see Gabbiani *et al.* 1994, for references). As the number of Golgi cells innervating a single granule cell and the efficacy of transmission is unknown, it is not possible to predict the precise relationship between Golgi cell firing rate and the frequency of sPSCs or the magnitude of G_{GABA}. Moreover, our estimates of G_{GABA} were obtained *in vitro* and at room temperature. Nevertheless, it seems likely that tonic activation of GABA$_A$ receptors will reduce significantly the excitability of granule cells when Golgi cell firing rates

are low and also enhance Golgi cell inhibition during periods of high-frequency discharge. Thus, in addition to phasic inhibition, arising from specific Golgi cell contacts, granule cells may experience a substantial tonic inhibition arising from activity at 'shared' synapses within the glomerulus, increasing further the divergence of Golgi cell output.

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